Biomedical Optics EXPRESS

Lowering photosensitizer doses and increasing fluences induce apoptosis in tumor bearing mice

Katja Haedicke,[1] Susanna Graefe,[2] Ulf Teichgraeber,[1] and Ingrid Hilger[1,*]

[1]*Experimental Radiology, Institute of Diagnostic and Interventional Radiology, Jena University Hospital, Friedrich-Schiller University Jena, Erlanger Allee 101, D-07747 Jena, Germany*
[2]*Biolitec Research GmbH, Research & Development, Otto-Schott-Straße 15, D-07745 Jena, Germany*
[*]*Ingrid.Hilger@med.uni-jena.de*

Abstract: The objective of this study was to determine an optimal dose of photodynamic therapy (PDT) for inducing apoptotic tumor cells in vivo. In this context, mice bearing human tongue-squamous epithelium carcinomas were treated with various photosensitizer concentrations and fluences. Tumor apoptosis was imaged after 2 days via a self-designed DY-734-annexin V probe using near-infrared fluorescence (NIRF) optical imaging. Apoptosis was verified ex vivo via TUNEL staining. Apoptotic tumor cells were detected in vivo at a dose of 40 µg photosensitizer and a fluency of 100 J/cm^2. This is the lowest photosensitizer dose reported so far.

OCIS codes: (170.0170) Medical optics and biotechnology; (000.1430) Biology and medicine; (170.0110) Imaging systems; (170.5380) Physiology.

References and links

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#262199 Received 30 Mar 2016; revised 19 May 2016; accepted 22 May 2016; published 16 Jun 2016

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1. Introduction

Photodynamic therapy (PDT) is a promising tumor treatment modality which uses laser light after administration of a light activatable substance, a so-called photosensitizer, in order to induce reactive oxygen species and thus cell death within the tumor [1]. Because of the non-invasiveness of the therapy, the non-toxicity of the used substances and light sources and the selective activability of the photosensitizer, PDT is of great interest nowadays. Due to the limited penetration depth of the laser light, PDT is, next to standard treatment regime like surgery or radiotherapy, particularly applied to treat tumors located near the surface of organs accessible to light, like head and neck cancer squamous cell carcinoma [2]. One adverse effect is that the skin is often highly photosensitive particularly after systemic administration of the non-specific, non-targeted photosensitizers [3], which is an additional strain for the respective cancer patients. For this reason it is of great interest to lower the dosages of the photosensitizers by concomitantly preserving the therapeutic effect. It is already known that tumor oxygenation and intratumoral inflammatory response, which cause cell death and thus tumor cure, depend on the conditions of PDT. In this context it is of interest that lower light fluence rates (< 50 mW/cm^2) or lower photosensitizer concentrations can improve PDT response and minimize side effects [4]. Furthermore, it is already described that lower fluence rates ($\mu W/cm^2$) caused more apoptosis in tumors and a more effective inhibition of growth of tumor cells in comparison to higher ones [5]. Nevertheless, particular knowledge on the optimal doses for photosensitizers and corresponding light doses to induce apoptotic tumor cells is lacking until now.

With consideration of the impact of PDT on tumor regression, apoptosis of target cells plays an important role [6]. Hereto, it was shown that it is possible to detect the efficacy of PDT at 2 days after therapy via multiplexed near-infrared fluorescence (NIRF) optical imaging of apoptotic tumor cells with a fluorescent annexin V probe and the tumor vascularization using an integrin targeting probe [7]. Nevertheless, the underlying molecular alterations after reduced photosensitizer concentrations and fluences of the laser light have never been investigated up to now.

Therefore, in this study we sought to investigate if there is an optimal dose concerning the photosensitizer concentration and the fluence of light during PDT which leads to the induction of apoptotic tumor cells and if this dose can be detected via NIRF optical imaging to early evaluate the treatment efficacy. After treating mice with different low dosed PDT, we used the annexin V probe for detecting apoptosis in vivo. Together with a self-designed ovalbumin probe we wanted to verify the specificity of this probe. We additionally proved the tumors ex vivo for the presence of apoptotic cells in order to further corroborate the in vivo imaging results. With this examination we expect to find a chance to enable imaging of the therapeutic efficacy of a low dose PDT very early after treatment.

#262199 Received 30 Mar 2016; revised 19 May 2016; accepted 22 May 2016; published 16 Jun 2016

2. Materials and methods

2.1 Cell line and animals

CAL-27 cells (tongue-squamous epithelium carcinoma cells, DSMZ) were maintained as a monolayer in DMEM-GlutaMAX (Gibco) with 10% FCS in a 5% CO_2 humidified atmosphere at 37 °C.

For in vivo experiments, 25 female athymic nude mice (Hsd:Athymic Nude-Foxn1nu (nu/nu), 20 - 25 g, Harlan Laboratories GmbH) were housed under standard conditions with food and water ad libitum. Xenografts were implanted subcutaneously by injecting 2 x 10^6 CAL-27 cells in Matrigel (BD) into the posterior back of 8 to 12 weeks old mice. The regional animal committee (Landesamt für Verbraucherschutz, Thuringia, Germany) approved the procedures. Experiments were in accordance with international guidelines on the ethical use of animals.

2.2 PDT of tumors

The photosensitizer formulation Foslip (liposomal formulation of meta-tetra(hydroxyphenyl)chlorin - mTHPC, on stage of development, Biolitec Research GmbH, Jena, Germany) was used for PDT. When the tumors reached a diameter of at least 5 mm, animals were divided into 5 groups. 2 groups (each n = 5) were injected intravenously with 20 and further 2 groups (each n = 5) with 40 µg Foslip per kg weight. 24 h later, tumors of these groups were illuminated with a 2 W 652 nm Ceralas PDT laser (Biolitec AG) with a fluence rate of 100 mW/cm^2, whereby one group with each respective Foslip dose was illuminated either with a fluence of 50 J/cm^2 (n = 5) or 100 J/cm^2 (n = 5). The fifth group was an untreated group (n = 5) without Foslip and without laser illumination to control the behavior of the tumors without therapy (Appendix, Fig. 3).

2.3 Coupling of annexin V and ovalbumin with the NIRF dyes

For imaging apoptotic tumor cells after PDT, annexin V (Abcam) was covalently coupled to the NIRF dye DY-734 (NHS-ester derivative, abs/em: 736/759 nm, Dyomics GmbH) as previously described [15]. The same protocol was used for coupling the NIRF dye DY-634 (NHS-ester derivative, abs/em: 635/658 nm, Dyomics GmbH) to ovalbumin (Hyglos GmbH).

2.4 Imaging of apoptotic tumor cells after PDT in vivo

For in vivo NIRF optical imaging of apoptotic tumor cells after PDT, DY-734-annexin V was injected intravenously at 2 days after therapy (8 nmol/kg weight, Fig. 3). Fluorescence intensity of the mice was monitored before injection of the probe (−1 h) as well as between 4 to 24 h thereafter with the Maestro in vivo fluorescence imaging system (excitation/emission filter: 670 - 710 nm/750 nm longpass, Cri Inc). Fluorescence intensity was analyzed semiquantitatively by spectral unmixing as described previously [7]. The fluorescence contrast from tumor to muscle was calculated by subtracting the average fluorescence signal of the muscle from that of the tumor.

To control the specificity of the binding of DY-734-annexin V, DY-634-ovalbumin was injected simultaneously into the same animals (20 nmol/kg weight, Fig. 3) and analysed correspondingly. Under this condition, the photosensitizer formulation Foslip was not excited (absorption/emission: 405/ 650 nm). Therefore, it is also not detected with the used filter settings, why it does not have to be considered in the imaging procedure.

2.5 Biodistribution of DY-734-annexin V and DY-634-ovalbumin probes

Animals of all groups were sacrificed at 3 days after PDT and 24 h after probe injection (Fig. 3). Biodistribution of the optical probes was analyzed by measuring the fluorescence

#262199 Received 30 Mar 2016; revised 19 May 2016; accepted 22 May 2016; published 16 Jun 2016

intensity of the tumors and the organs ex vivo as described above. Tumors were frozen in liquid nitrogen and stored at −80 °C for histological analysis.

2.6 Imaging of apoptotic tumor cells after PDT ex vivo

Fixed cryo-frozen tumor slices (the middle of the tumor) were stained with terminal deoxynucleotidyl transferase (TdT) dUTP nick end labelling (TUNEL) to detect DNA fragmentations as a marker of apoptotic tumor cells ex vivo. Slices were processed as mentioned by the manufacturer (TACS 2 TdT-Fluor In Situ Apoptosis Detection Kit, Trevigen Inc.).

2.7 Statistical analysis

Data are expressed as means ± standard error of mean. Statistical significance was analyzed by a two-tailed Student's t test or if failing by a Mann-Whitney Rank Sum test using SigmaPlot 12.0. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Characterization of DY-734-annexin V and DY-634-ovalbumin

The absorption spectrum revealed a high peak at 717 nm and 621 nm for DY-734-annexin V and DY-634-ovalbumin respectively (Fig. 3). Both spectra also showed a smaller peak of the respective protein at 278 nm. The dye to protein ratio yielded 0.3 to 0.5 DY-734 molecules per annexin V protein and 0.4 to 0.6 DY-634 molecules per ovalbumin protein. To prevent changes of the two probes in tumor enrichment as a result of influences by the dyes, fluorophores with a similar chemical structure were selected (Fig. 3).

3.2 Detection of an optimal dose of PDT via imaging of apoptosis in vivo

Using NIRF optical imaging, apoptosis could only be detected within the tumors if they were previously treated with a therapeutic dose of 40 µg Foslip per kg weight and a fluence of 100 J/cm^2. This was shown via a significantly ($p < 0.05$) higher fluorescence contrast of DY-734-annexin V in this animal group compared to the lower dosed groups (20 µg Foslip per kg weight and 50 J/cm^2) as well as to the untreated control group, especially at a time frame from 6 up to 24 h after probe injection (Fig. 1(A)). Only a very low fluorescence signal could be observed from the tumors of the other animal groups. None of the animals exhibited alterations in the skin after treatment with PDT. Nearly no accumulation could be seen for DY-634-ovalbumin in all tumors (Fig. 1(B)).

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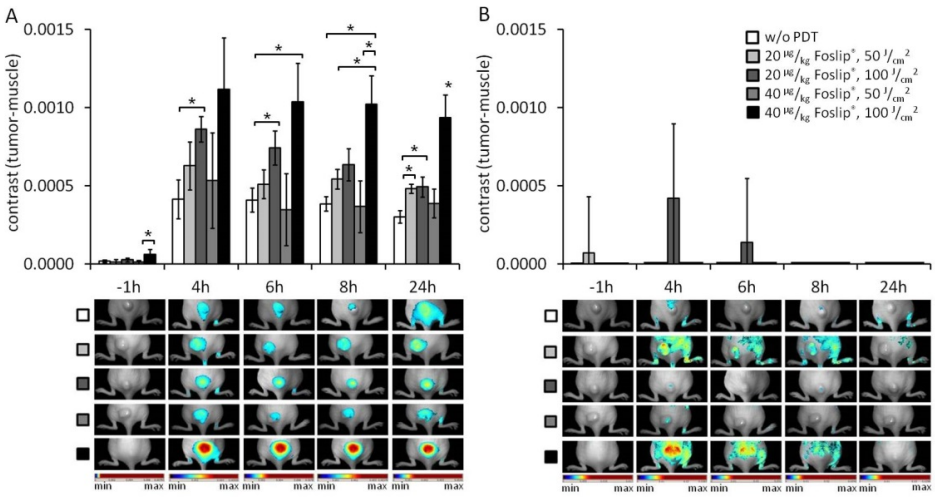

Fig. 1. Detection of PDT-induced apoptotic cells in tumors at 2 days after treatment at the highest therapeutic dose (40 µg/kg Foslip, 100 J/cm^2) and verification of the specificity of this detection in vivo. Fluorescence contrast of DY-734-annexin V (A) or the DY-634-ovalbumin probe (B) between CAL-27-tumors and muscle of untreated (white bars) and different dosed PDT-treated mice at 2 days after therapy up to 24 h post injection of the probe (upper rows). Illustrations show representative composite fluorescence images of CAL-27 tumor bearing mice (lower rows). Each group n = 5, *p < 0.05.

3.3 Biodistribution of DY-734-annexin V and DY-634-ovalbumin

Biodistribution analysis of DY-734-annexin V (3 days after PDT and 24 h after probe injection) showed the highest fluorescence signal in the kidneys of all PDT-treated animals, (Fig. 4). Comparing the fluorescence of the tumors, the highest fluorescence intensity was seen in the highest dosed treated tumors although the difference is not very pronounced. Almost no signal was detected in the heart, spleen, and lung of all groups. In comparison, biodistribution of the DY-634-ovalbumin probe showed only a distinct accumulation in the stomach (Fig. 4).

3.4 Detection of an optimal dose of PDT via imaging of apoptosis ex vivo

After staining tumor slices with TUNEL, apoptotic cells could only be detected within the tumors of animals treated with the highest dose (40 µg Foslip per kg weight and 100 J/cm^2; green in Fig. 2).

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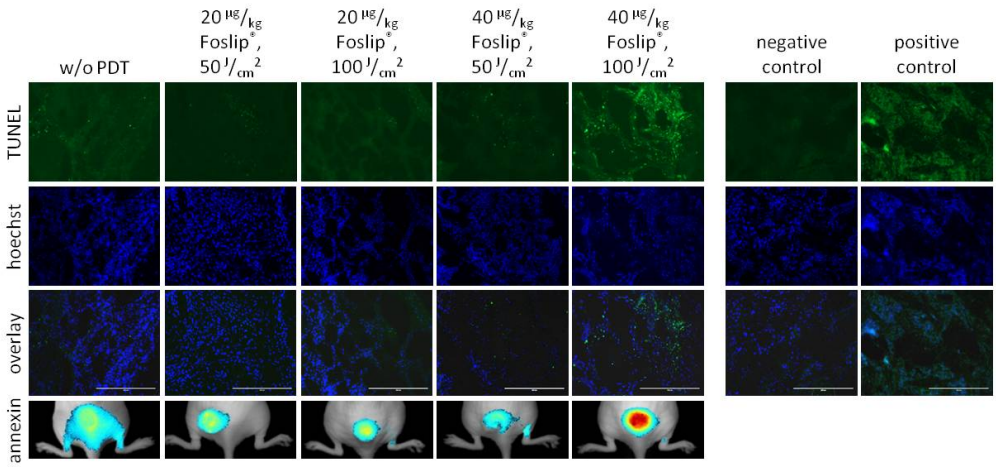

Fig. 2. TUNEL staining of apoptotic cells in the highest investigated dose of PDT (40 µg/kg Foslip, 100 J/cm^2) coincides with in vivo imaging. Green: apoptotic cells (TUNEL). Blue: cell nuclei (hoechst). Negative control: TUNEL staining without TdT-enzyme. Positive control: TUNEL staining with TACS-nuclease. Bar: 200 µm. Lower Row: representative composite images 24 h after injection of DY-734-annexin V in CAL-27 tumor bearing mice.

4. Discussion

In our study we were able to show that an optimal dose for the photosensitizer as well as for fluence of light is required to achieve tumor regression upon treatment with low doses of PDT (40 µg Foslip per kg weight, 100 J/cm^2). Furthermore, the therapeutic efficacy of such a low dose PDT can be detected already at early time points upon treatment and when small molecular alterations of tumor cells begin to rise. Consequently, this improves the discrimination between an effective and an ineffective treatment.

We assume that the delivered doses of drug and light are equivalent to those taken up by the tissue since we used 1) laser light (652 nm) with good feasibility for tissue penetration, and 2) subcutaneous tumors up to 8 mm with good accessibility.

The detection of the therapeutic effects via NIRF optical imaging was reliable, since a clear fluorescence signal in the tumors was perceived with the DY-734-annexin V probe. A clear signal was only visible using the highest dose of PDT (40 µg Foslip per kg weight, 100 J/cm^2), indicating that the probe is specifically retained only in these tumors due to the presence of phosphatidylserine on the surface of apoptotic tumor cells. Indeed the occurrence of apoptosis due to PDT was already confirmed by the nuclei staining of tumor cells after PDT and the occurrence of condensed chromatin (equivalent drug doses) [7].

The fluorescence signal emerging from the annexin V probe was specific, since nearly no fluorescence signal could be detected for the DY-634-ovalbumin control probe in all animal groups, in spite of injecting a more than two-fold higher dose of this control protein (20 nmol/kg weight) compared to annexin V (8 nmol/kg weight). This observation demonstrates the high specificity of DY-734-annexin V.

The detection of intratumoral apoptosis was only possible after application of the highest dose with 40 µg Foslip per kg weight and 100 J/cm^2. Lower dosages showed fluorescence signals comparable to untreated tumors. These results could be corroborated using TUNEL staining of the tumor slices ex vivo. In comparison, other investigations mostly used higher concentrations (e.g. fluency of > 100 mW/cm^2, photosensitizer concentration of 300 or 500 µg mTHPC per kg weight (e.g [8–10].)). The usage of a lower dose of mTHPC in our study is possible due to its encapsulation in liposomes, which reduced the unspecific distribution and accumulation of the drug in the body and thus increased its therapeutic efficacy. In

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previous studies, the liposomal formulation Foslip was injected with 150 or even 300 µg of mTHPC per kg weight, meaning almost four to eight times more drug than we used [11, 12].

Therefore, our data show that the amount of the photosensitizer could be considerably reduced without losing the therapeutic efficacy. This strategy would lower the side effects for patients by minimizing the photosensitivity of the skin and thus leads to a more comfortable lifestyle for patients after therapy. Another advantage is that lower dose rates of light prevent photobleaching of the photosensitizer and do not deplete oxygen [13] what makes PDT reaction more efficient because of the sufficient availability of oxygen for the drug.

With consideration of the comparatively high degree of apopotosis found in tumors (i.e.40 mg/kg Foslip and 100 mW/ cm^2), one can assume that under our conditions the availability of intralesional ground-state oxygen was sufficient in order for the therapy to be effective. In particular, photochemical oxygen consumption in the tumor area is proportional to the light fluence, the local concentration of the photosensitizer and its absorption coefficient [14].

Furthermore, we observed that the liposomal photosensitizer formulation Foslip tends to show a dose dependency regarding the therapeutic efficacy, since an effect could be seen with 40 µg Foslip per kg weight but not with 20 µg per kg weight after illumination with a fluence of 100 J/cm^2. Therefore, a dose dependency might not only be applicable for the pure photosensitizer mTHPC [8] but also for its liposomal formulation Foslip.

The effects of low dose PDT could be efficiently and systematically monitored in our study via NIRF optical imaging. Until now this effect has been merely visualized in vitro or ex vivo after sacrificing the animals and in vivo only by monitoring necrotic cell death after near-infrared photoimmunotherapy by means of fluorescence lifetime imaging [15]. Since our animals did neither exhibit any alterations of the skin nor any other unusual changes, we propose that NIRF optical imaging is an appropriate method for non-invasive and selective imaging of therapy PDT responses.

With regard to the biodistribution analysis of DY-734-annexin V, a rough correlation of the biodistribution of the annexin V probe could be observed for the two different fluences of light but not for the different Foslip concentrations in kidney and liver. The reason for this effect is still unknown but it seems to be PDT- or rather light-specific because in another study the application of chemotherapy did not interfere with the quantitative uptake of radiolabelled annexin V in normal human tissues [16]. Another point is that the control probe DY-634-ovalbumin did not show this light dependent pattern, even though this probe had a similar molecular weight as the annexin V probe and both coupled dyes showed nearly the same chemical structure.

5. Conclusion

We could show that low dose PDT by application of 40 µg Foslip per kg weight and a fluence of 100 J/cm^2 distinctly induces apoptosis in tumors. Our results contribute to the selection of appropriate therapeutic parameters for clinical evaluation and enable an efficient application of resources by simultaneous reduction of unwanted side effects, such as skin photosensitivity. We opened up new ways to minimize this side effect by reducing the photosensitizer dose to a minimum.

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Appendix

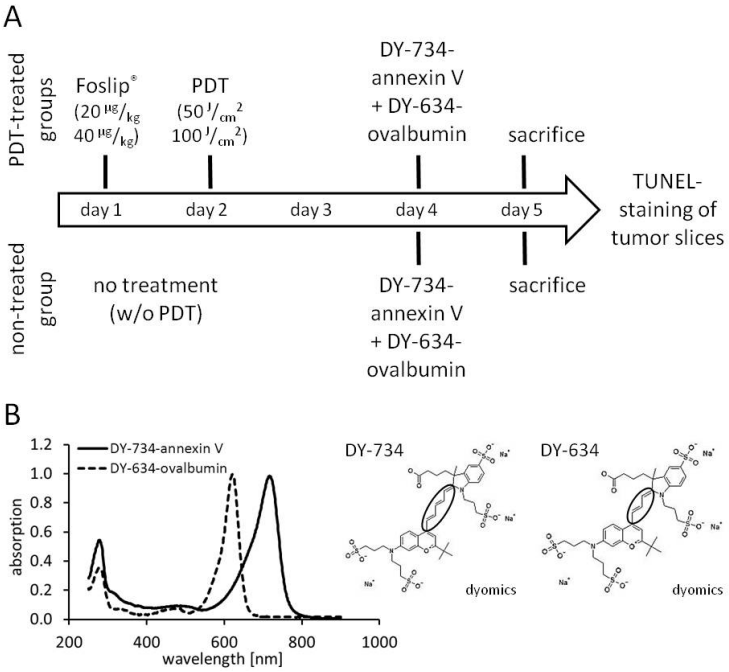

Fig. 3. Timeline of the study and absorption spectrum of the used probes. A: Experimental design. DY-634-ovalbumin was used as a specificity control for the DY-734-annexin V probe. B: Absorption spectrum of DY-734-annexin V and DY-634-ovalbumin and chemical structures of the dyes.

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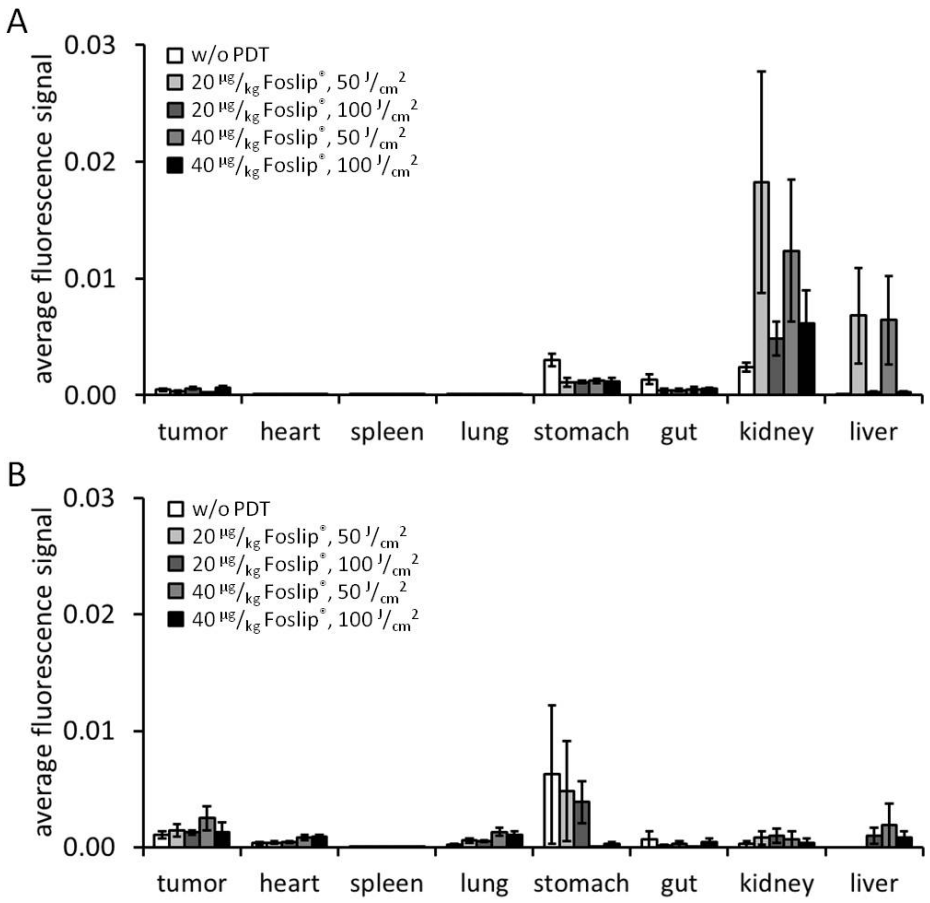

Fig. 4. Biodistribution of DY-734-annexin V (A) and DY-634-ovalbumin (B) probe 24 h after probe injection in CAL-27 tumor bearing mice treated or not with PDT.

Acknowledgements

The project was supported by the Thüringer Aufbaubank (Erfurt, Germany). We gratefully acknowledge Susann Burgold and Yvonne Ozegowski for valuable technical assistance in animal handling and Julia Göring for preparation of cryo-frozen tumor slices.